GENETIC SCREENING FOR IDENTIFICATION OF *SALMONELLA TYPHIMURIUM* TN5 MUTANTS WITH POTENTIAL HYPERSENSITIVITY TO SHORT-CHAIN FATTY ACIDS

Y.M. KWON[1, 2], L.F. KUBENA[2], D.J. NISBET[2] and S.C. RICKE[3,4]

[2]*USDA-ARS*
Southern Plains Agricultural Research Center
College Station, TX 77845

[3]*Department of Poultry Science*
Texas A & M University
College Station, TX, 77843

Accepted for Publication May 15, 2003

ABSTRACT

This study was conducted to test if transposon footprinting could be used to identify transposon mutants of Salmonella typhimurium *with growth defects in a media containing short-chain fatty acids (SCFA) as the test selective condition. High concentrations of SCFA are one of the characteristic conditions in the animal intestine that has been suggested to play a role in inhibiting colonization by nonindigenous bacterial pathogens. When the mutant pools containing 25 Tn5 mutants/pool were analyzed for transposon footprints before and after selection, a polymerase chain reaction (PCR) product could be identified that was present in an input pool, but not in a corresponding output pool. The results indicate that transposon footprinting can be used for negative screening of genes sensitive to SCFA in the* S. typhimurium *bacterial genome.*

INTRODUCTION

Natural infection of poultry by foodborne *Salmonella* spp. occurs via the oral route and salmonellae colonize the intestinal tract with the crop and ceca being the primary sites of colonization (Brownwell *et al.* 1970; Soerjadi *et al.*

[1] Current Address: Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR, 72701

[4] Correspondence to: Dr. Steven Ricke, Poultry Science Department, 2472 TAMU, College Station, TX 77843-2472. TEL: (979) 862-1528; FAX: (979) 845-2377; EMAIL: sricke@poultry.tamu.edu

Journal of Rapid Methods and Automation in Microbiology 11 (2003) 89–95.

1981; Stavric 1987; Impey and Mead 1989). However, despite considerable evidence that establishment of indigenous microflora and their continued presence in the gut are essential for preventing pathogen colonization, little is known about the mechanism(s) involved.

Previously, we (Durant *et al.* 2000; Kwon and Ricke 1998a, b; Kwon *et al.* 1998; Kwon *et al.* 2000) have established that some short-chain volatile fatty acids (SCFA) are more inhibitory to salmonellae than others under anaerobic growth conditions and that survival response of salmonellae to SCFA differs for the same SCFA depending on whether the culture is growing anaerobically or aerobically. This has implications not only for gastrointestinal ecology competitiveness of salmonellae but in the case of SCFA raises practical issues regarding the use of organic acids as an intervention step in poultry processing (Kwon *et al.* 2000). Issues to consider include whether anaerobic or aerobic growth predisposes salmonellae to be more resistant to particular organic acids and how these growth conditions might influence virulence and pathological characteristics of salmonellae (Durant *et al.* 2000; Kwon and Ricke 1998a, b; Kwon *et al.* 1998; Kwon *et al.* 2000).

Kwon and Ricke (2000) devised an efficient PCR-based method for specific amplification of transposon-flanking sequences. This method requires the sequence information of only transposon-specific sequences, consists of two simple steps of ligation and amplification and does not exhibit nonspecific background amplification. It can amplify multiple independent insertions present either within a mutant or in a pool of multiple mutants. This method, termed transposon footprinting, simultaneously amplifies the Tn*5*-flanking sequences in a complex pool of the Tn*5* mutants. As the length of the amplified DNA fragment should be unique for distinct Tn*5* mutant, the PCR products separated on an agarose gel generate transposon footprint with each band in the footprint representing the corresponding Tn*5* mutant. Our overall goal in this study was to determine whether this simple and rapid method could be applied to identify transposon mutants exhibiting a growth defect in the presence of a SCFA concentration typically encountered by foodborne *Salmonella* spp. in the gastrointestinal tract.

MATERIALS AND METHODS

Transposon Mutagenesis

Escherichia coli SM 10 λ *pir* transformed with the suicide plasmid pUT/Km was used to generate mini-Tn*5* mutants of *S. typhimurium* ATCC 14028 that had been selected for resistance to nalidixic acid as described by Herrero *et al.* (1990). Briefly, the donor cells, *E. coli* SM 10λ *pir* carrying the plasmid pUT/Km were mixed with recipient cells, *Salmonella enterica* serotype Typhimurium (*S. typhimurium*) with resistance to nalidixic acid, on Luria-Bertani (LB; Difco Laboratories, Detroit, MI) plated and incubated overnight at 37C. After incubation, the cells were recovered in 1 mL phosphate buffered saline (PBS, pH 7.2) and plated on LB plates containing kanamycin (75 μg/mL)and nalidixic acid (25 μg/mL). The resulting colonies on the plates after overnight incubation at 37C were considered the recipient cells with transposon inserted into the chromosome.

Preparation of Input and Output Pools of Tn5 Mutants

Each pool of *S. typhimurium* 14028s, each of which consisted of 25 randomly chosen Tn5 mutants, was inoculated to aerobic E medium (A), anaerobic E medium (B), and anaerobic E medium containing SCFA mixture (70 mM acetate, 25 mM propionate, 26 mM butyrate; C), and incubated at 37C for 12 h. Anaerobic media were prepared using an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) containing an anaerobic atmosphere (10% H_2 10% CO_2, 80% N_2). Genomic DNA was isolated from the pool used for inoculation (input pool) and the mutant pool recovered after growth in selective media A, B and C (output pool) using QIAamp DNA mini kit (Qiagen Inc., Carlsbad, CA) and used to generate transposon footprints.

Preparation of Template DNA for PCR

The oligonucleotide sequences used in this study was described previously (Kwon and Ricke 2000). A 9 μL of linker 2 (350 ng/μL) was first phosphorylated at the 5' end using T4 polynucleotide kinase (PNK). After heat denaturation of PNK at 65C for 20 min, 9 μL of linker 1 (350 ng/μL) was added to a final volume of 29 μL. The mixture of linkers 1 and 2 were heated to 95C for 2 min and cooled slowly to room temperature to allow annealing. Bacterial genomic DNA isolated from each pool was completely digested with *Nla*III (New England BioLabs, Beverly, MA). Approximately 40 ng of the digested DNA was ligated to 1 μg of the Y linker with 1 μL of T4 DNA ligase (1 unit/μL; Invitrogen, Carlsbad, CA) in a final volume of 20 μL. After overnight incubation at room temperature, the reaction mixture was diluted with double distilled water to a final volume of 200 μL and heated at 65C for 10 min to denature T4 DNA ligase. Subsamples (2 μL) were used as templates in the PCR amplification.

PCR Amplification and Gel Electrophoresis

All PCR reactions were performed using GeneAmp PCR system 2400 (PE Applied Biosystems, Foster City, CA) along with a primer specific to Tn5 (Tn5 primer) and a primer specific to the Y linker (Y linker primer). The Tn*5* primer was designed to specifically anneal to the I end of the Tn5 (Herrero *et al.* 1990; Auerswald *et al.* 1981). The reaction containing 5 μL 10X PCR buffer (166 mM $(NH_4)_2SO_4$, 670 mM Tris (pH 8.8), 67 mM $MgCl_2$, 100 mM ß-mercaptoethanol), 350 ng of each primer, 3 μL dNTPs mix (25 mM for each dNTP), 3 μL DMSO, and 2 μL template DNA in a 49 μL reaction mixture was incubated at 95C for 2 min. As a next step, 1 μL Taq DNA polymerase (5 units/μL; PE Applied Biosystems) was added during a hot-start incubation at 80C to prevent nonspecific priming. The target sequences were amplified through 30 cycles of 95C for 30 s, 58C for 1 min, and 70C for 1 min, followed by a final cycle of 70C for 5 min. The PCR products were analyzed on a 1.5% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

Salmonella spp. can encounter various environmental stress conditions, such as nutrient starvation, pH extremes, oxidative stress, osmotic shock, and heat shock during its infectious life cycle (Foster and Spector 1995). Those stress conditions may have dramatic effect(s) on growth, survival and potentially on virulence of the pathogen (Archer 1996). One of the potential stress conditions that can be frequently encountered by *S. typhimurium* is short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate. SCFA are produced as fermentation products by native intestinal microflora and can be present at high concentrations in gastrointestinal ecosystems possessing large numbers of highly fermentative anaerobic bacteria. In humans, the concentration of SCFA is 35 mM/kg in small intestine and 134 mM/kg in the large intestine (Cummings *et al.* 1987). However, the genetic factors effecting the growth or survival of *S. typhimurium* in the presence of SCFA have not been described.

In an effort to understand the mechanism by which *S. typhimurium* is affected by SCFA in animal intestine, we applied transposon footprinting to identify the genes that are required for growth of *S. typhimurium* in the presence of SCFA at the concentrations typically found in the animal intestine. We used minimal media (E medium), anaerobic E medium, and anaerobic E medium containing SCFA (70 mM acetate, 25 mM propionate, 26 mM butyrate) as selective conditions to represent the gastrointestinal tract concentration of SCFA under anaerobic atmospheric conditions.

Each pool of 25 Tn*5* mutants was inoculated to the selective media and the mutant cells were recovered after growth to stationary phase. The mutant pool that was used for inoculation (input pool) and the mutant pools that were recovered after selection (output pool) were used for isolation of genomic DNA, which was subsequently used to generate transposon footprints as described in the Materials and Methods section.

The unique transposon footprints obtained from two Tn*5* mutant pools (A and B) by separating the amplified Tn*5*-flanking sequences on a 1.5% agarose gel are shown in Fig. 1. In pool B, the pattern of transposon footprints are conserved among the input and three output pools, indicating that none of the mutants in the pool B exhibited a growth defect in the selective conditions. However, the results obtained from pool A show that one of the bands (indicated by an arrow in Fig. 1) is missing only in the anaerobic E media containing SCFA. Based on these results it is apparent that the mutant corresponding to the missing band was not recovered from the selective condition and it is likely that the mutant possessed a growth defect when cultured in anaerobic E media containing SCFA.

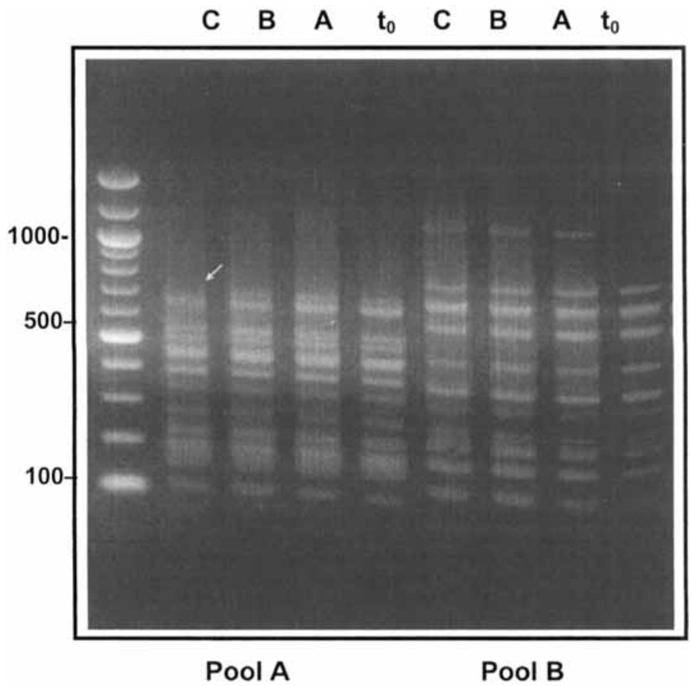

FIG. 1. TRANSPOSON FOOTPRINTS OF *S. TYPHIMURIUM* TN5 MUTANT POOLS BEFORE (t_0) AND AFTER GROWTH IN E MEDIUM, ANAEROBIC E MEDIUM, AND ANAEROBIC E MEDIUM CONTAINING SCFA MIXTURES

A 100 bp DNA ladder (New England BioLabs) was used as a standard marker. Transposon footprints were generated from input pool (t_0) and output pools recovered from aerobic E medium (A), anaerobic E medium (B), and anaerobic E medium containing SCFA mixture (70 mM acetate, 25 mM propionate, 26 mM butyrate; C).

The successful application of transposon footprinting method has been used previously to rapidly screen mutants for potential genes required for survival during environmental stress that occurs under nongrowth conditions (Park *et al.* 2002; Kwon *et al.* 2003). In the current study, this approach also appears to be useful for rapidly identifying hypersensitive *S. typhimurium* mutants after selective growth in the presence of high concentrations of SCFA characteristic of the extensive fermentation occurring in the gastrointestinal tract.

ACKNOWLEDGMENTS

This research was supported by Hatch grant H8311 administered by the Texas Agricultural Experiment Station and USDA-NRI grant number 2002-35201-11608.

REFERENCES

ARCHER, D.L. 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. Trends Food Sci. Technol. *7*(3), 91-95.

AUERSWALD, E.A., LUDWIG, G. and SCHALLER, H. 1981. Structural analysis of Tn*5*. Cold Spring Harbor Symp. Quant. Biol. *45*, 107–113.

BROWNWELL, J.R., SADLER, W.W. and FANELLI, M.J. 1970. Role of the ceca in intestinal infection of chickens with *Salmonella* typhimurium. Avian Dis. *14*(1), 106–116.

CUMMINGS, J.H., POMARE, E.W., BRANCH, W.J., MAYLOR, C.P.E. and MACFARLANE, G.T. 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. *28*(10), 1221–1227.

DURANT, J.A., CORRIER, D.E. and RICKE, S.C. 2000. Short-chain volatile fatty acids modulate the expression of the *hilA* and *invF* genes of *Salmonella* Typhimurium. J. Food Prot. *63*(5), 573–578.

FOSTER, J.W. and SPECTOR, M.P. 1995. How *Salmonella* survive against the odds. Ann. Rev. Microbiol. *49*, 145–174.

HERRERO, M., DE LORENZO, V. and TIMMIS, K.N. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. *172*(11), 6557–6567.

IMPEY, C.S. and MEAD, G.C. 1989. Fate of salmonellas in the alimentary tract of chicks pre-treated with a mature caecal flora to increase colonization resistance. J. Appl. Bacteriol. *66*(6), 469–475.

KWON, Y.M., HA, S.D. and RICKE, S.C. 1998. Growth response of a *Salmonella typhimurium* poultry isolate to propionic acid in aerobic and anaerobic growth conditions. J. Food Safety *18*(2), 139–149.

KWON, Y.M., KUBENA, L.F., NISBET, D.J. and RICKE, S.C. 2003. Isolation of *Salmonella typhimurium* Tn*5* mutants defective for survival on egg shell surface transposon footprinting. J. Environ. Sci. *B38*(1), 103–109.

KWON, Y.M., PARK, S.Y., BIRKHOLD, S.G. and RICKE, S.C. 2000. Induction of resistance of *Salmonella typhimurium* to environmental stresses by exposure to short-chain fatty acids. J. Food Sci. *65*(6), 1037–1040.

KWON, Y. M. and RICKE, S C. 1998a. Survival of a *Salmonella typhimurium* poultry isolate in the presence of propionic acid under aerobic and anaerobic conditions. Anaerobe *4*, 251–256.

KWON, Y.M. and RICKE, S.C. 1998b. Introduction of acid resistance of *Salmonella* typhimurium by exposure to short-chain fatty acids. Appl. Environ. Microbiol. *64*(9), 3458-3463.

KWON, Y. M. and RICKE, S.C. 2000. Efficient amplification of multiple transposon-flanking sequences. J. Microbiol. Meth. *41*, 195–199.

PARK, S.Y., KWON, Y.M., BIRKHOLD, S.G., KUBENA, L.F., NISBET, D.J. and RICKE, S.C. 2002. Application of a transposon footprinting technique for rapid identification of *Salmonella typhimurium* Tn*5* mutants required for survival under desiccation stress conditions. J. Rapid Methods Automation Microbiology *10*(3), 197-206.

SOERJADI, A.S., STEHMAN, S.M., SNOEYENBOS, G.H., WEINACK, O. M. and SMYSER, C.F. 1981. Some measurements of protection against paratyphoid Salmonella and Escherichia coli by competitive exclusion in chickens. Avian Dis. *25*(3), 706–712.

STAVRIC, S. 1987. Microbial colonization control of chickens intestine using defined cultures. Food Technol. *41*(7), 91–98.